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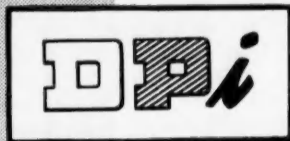
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APPLICATION OF PAPER CHROMATOGRAPHY TO ORGANIC CHEMISTRY

By **STANLEY R. AMES***

Paper chromatography is one of the most versatile analytical procedures yet devised for the separation of mixtures of organic compounds. It is a major development in modern analytical chemistry. Closely related members of such diverse classes as inorganic ions, amino acids, fatty acids, carbohydrates, proteins, steroids, vitamins, and antibiotics have been successfully separated by paper chromatography.

What is paper chromatography? A beautifully concise definition of the scope of chromatography has been devised by Strain (1): "All chromatographic methods are based upon the differential migration of solutes through polyphase systems in which the phases have preferential affinities for the solutes." There are three general classes of chromatography. In "partition chromatography," the solutes are distributed between two solvent systems, one of them moving with respect to the other. Separations involving partition chromatography may be likened to a series of separatory funnels, in which one solvent remains fixed and the other solvent is transferred continuously from one funnel to the next. Separations based solely on partition of solutes between two immiscible solvents have reached technical excellence as "countercurrent distribution." "Adsorption chromatography" usually involves a liquid phase moving past a solid phase which has preferential affinities for the solutes. This may be likened to a series of successive distributions of solutes between a solvent and a solid adsorbent. The less strongly adsorbed material will be eluted before those more strongly adsorbed. In "ion-exchange chromatography," the adsorption of the solute on the solid phase depends specifically on a chemical reaction involving interchange of ions. A familiar example of ion exchange is the softening of hard water with a zeolite resin. The less strongly adsorbed sodium ions are replaced on the resin by the more strongly bound calcium and magnesium ions.

Any chromatographic procedure utilizing paper as the stationary phase or as the support for the stationary phase falls within the scope of paper chromatography. Chromatographic separation on paper usually depends mainly on partition, but adsorption and ion exchange are involved to varying degrees.

The earliest record of paper chromatography was the description by Pliny of the use of papyrus impregnated with an extract of gallnuts for the detection of ferrous sulfate (2). About 1860, Schönbein, and later Goppelsroeder, employed paper for

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the separation of mixtures of dyes in aqueous solutions. This procedure, "Kapillar-analyse," depended solely on adsorption and did not involve a second solvent. The advent of modern paper-partition chromatography was the now classic report by Consden Gordon, and Martin in 1944 (3). Since that time, paper chromatography has been successfully applied to the separation of many complex, naturally occurring and synthetic mixtures.

Techniques of Paper Chromatography

A typical application of paper chromatography, and probably the most common one, is the separation of a mixture of amino acids. The technique may be summarized as follows: A strip of filter paper (2 x 45 cm.) serves as the support. A small volume (5-20 microliters) of a solution of the amino-acid mixture (10-200 micrograms of amino acids) is spotted about 4 cm. from one end of the paper strip. A cylindrical glass jar about 18-24 in. high, covered with a glass plate, is often employed as the chromatographic chamber. Two beakers are placed on the bottom of the chamber, one containing *n*-butanol saturated with water and the other containing water saturated with *n*-butanol. This is to ensure equilibrium between the vapor and liquid phases and to avoid distortion of the chromatogram. A trough containing the solvent phase is supported near the top of the chamber. The strip is placed in the chamber with the starting end (the one nearest the spot of amino-acid mixture) dipping into the solvent phase (*n*-butanol saturated with water). This developing solvent is permitted by capillary action to move down the strip. The water adheres to the hydrated paper support to a greater extent than the less polar *n*-butanol. As the solvent front moves by the test spot, the amino acids present migrate at rates dependent on their relative solubilities in *n*-butanol and in water. The less polar amino acids will be more soluble in the *n*-butanol phase and therefore will migrate more rapidly than the more polar ones. After a period of 18-24 hours, the solvent front will have migrated almost to the bottom of a 45-cm. strip, and the mixture will have separated into its individual components. The strip is removed from the chamber and dried. Spraying with 1,2,3-triketohydrindene (ninhydrin) leads to the formation of a series of colored spots, each of which indicates a separate amino acid. The distance that a given amino acid has migrated divided by the distance that the solvent front has moved is termed the R_f value. Identification of the individual amino acids can be made by the color of the complex and by the R_f value, which is a characteristic constant for a given substance under a given set of conditions. The procedure can be made quantitative by colorimetric analysis of extracted spots, or semiquantitative by estimating the size and depth of color of the spot on the paper.

In "two-dimensional chromatography," this basic procedure has been extended by employing a rectangular sheet of filter paper. The test mixture is spotted near one corner. The sheet is developed with the first solvent (such as phenol:water) in one direction. After drying, the sheet is rotated 90°, with the edge nearest the amino acids inserted into a second developing solvent (such as 2,4-lutidine:2,4,6-collidene:water). Thus, the second solvent is flowed onto the sheet in a direction perpendicular to the direction of flow of the first solvent. The resulting two-dimensional chromatogram gives almost complete resolution of complex mixtures such as protein hydrolyzates.

The basic procedure for paper chromatography can be modified further, depending on the direction of solvent flow. In "descending" chromatography, the solvents flow downward, capillary attraction working in conjunction with gravity. When the solvent flows upward, gravity opposing capillary rise, it is termed "ascending" chromatography. The latter procedure has the advantage of simplicity of apparatus

and adaptability to a large number of samples. However, the flow of the solvent front is retarded, leading to inconsistencies during prolonged runs.

Developing Solvents

Every component of a mixture has a characteristic position on a paper chromatogram. This position depends on the chemical structure of the component, the polarity of the supporting matrix (paper), and the solvent system employed. First let us consider the effect of the composition of the solvent system. *n*-Butanol saturated with water is a common developing solvent. Following development with this solvent, the very polar substances, for example, methyl glyoxal, will be found at the starting point. Very nonpolar substances, for example, triolein, will be found coincident with the advancing solvent front. Use of a more nonpolar solvent, such as *n*-amyl alcohol saturated with water, results in a greater concentration of polar material at the origin and gives a better resolution of the more nonpolar components. Conversely, use of a more polar solvent system, such as pyridine:acetic acid:water, results in greater resolution of the polar components and poorer resolution of the nonpolar constituents. The relative polarity of various solvents can often be estimated from their dielectric constants.

Hydrogen-bonding is of great importance in determining the R_f value. Solvents capable of forming hydrogen bonds thus effect good resolution. Phenol, an excellent proton donor, is an excellent solvent for resolving compounds containing proton acceptors, such as amino groups. Compounds containing proton donors (hydroxyl groups) are best resolved employing a solvent that is a proton acceptor, such as 2,4,6-collidine. Other factors affecting the partition coefficient will similarly change the R_f value. In applying these techniques to the separation of mixtures, the analyst's main problem is to determine the conditions effecting the best resolution.

Reversed-Phase Paper Chromatography

Many mixtures of organic compounds can be separated by using cellulose filter paper with an organic solvent saturated with water for developing the chromatogram (see accompanying table). However, this type of system is not satisfactory for relatively nonpolar compounds. Such materials will migrate with the advancing solvent front, and no resolution will be effected. The inherent difficulty of separating nonpolar materials on a highly polar support like paper has resulted in the development of "reversed-phase chromatography." In this modification, paper has been impregnated with paraffin, silicones, or rubber latex to make it less polar. By this means, the support now has a greater affinity for the less polar solvent. The nonpolar solvent (*n*-butanol) now becomes the stationary phase and the polar solvent (water) is the moving phase, hence the term reversed-phase chromatography. Alternatively, two immiscible organic solvents can be employed with the more polar one as the moving phase.

The use of reversed-phase chromatography makes it possible to separate mixtures of nonpolar organic compounds. The strong hydrogen-bonding, hydration, and ion-exchange forces prevailing in solvent:water:cellulose systems are no longer present. Separations can result from differences in weak hydrogen-bonding or even Van der Waals forces. With such weak forces between solvent and solute prevailing, affinity of the paper support for the solute becomes a critical factor. Separations in reversed-phase chromatography may thus be in part due to adsorption.

Examples of reversed-phase chromatography are the separation of fatty acid esters on paper impregnated with rubber latex using a methanol:acetone developing solvent, and steroids using silicone-treated paper with benzene:chloroform

as the developing solvent. Recently, filter papers composed of fibers of cellulose esters or various polymers have been prepared. Rapid advances in reversed-phase chromatography are anticipated.

Identification

There are numerous means of identification of the components of a mixture after separation by paper chromatography. Generally speaking, any distinctive property of a compound or of a derivative of a compound can be adapted to paper chromatography. The component can either be identified on the chromatogram or following elution from the paper. Of course, if the compound is colored, there is no problem. Frequently, a colored derivative can be formed on the paper by spraying with the appropriate reagent. Measurements of the absorption characteristics in the visible, ultraviolet, or infrared regions can be utilized. Biological function or radioactive tracers have been employed for identification.

Evaluation

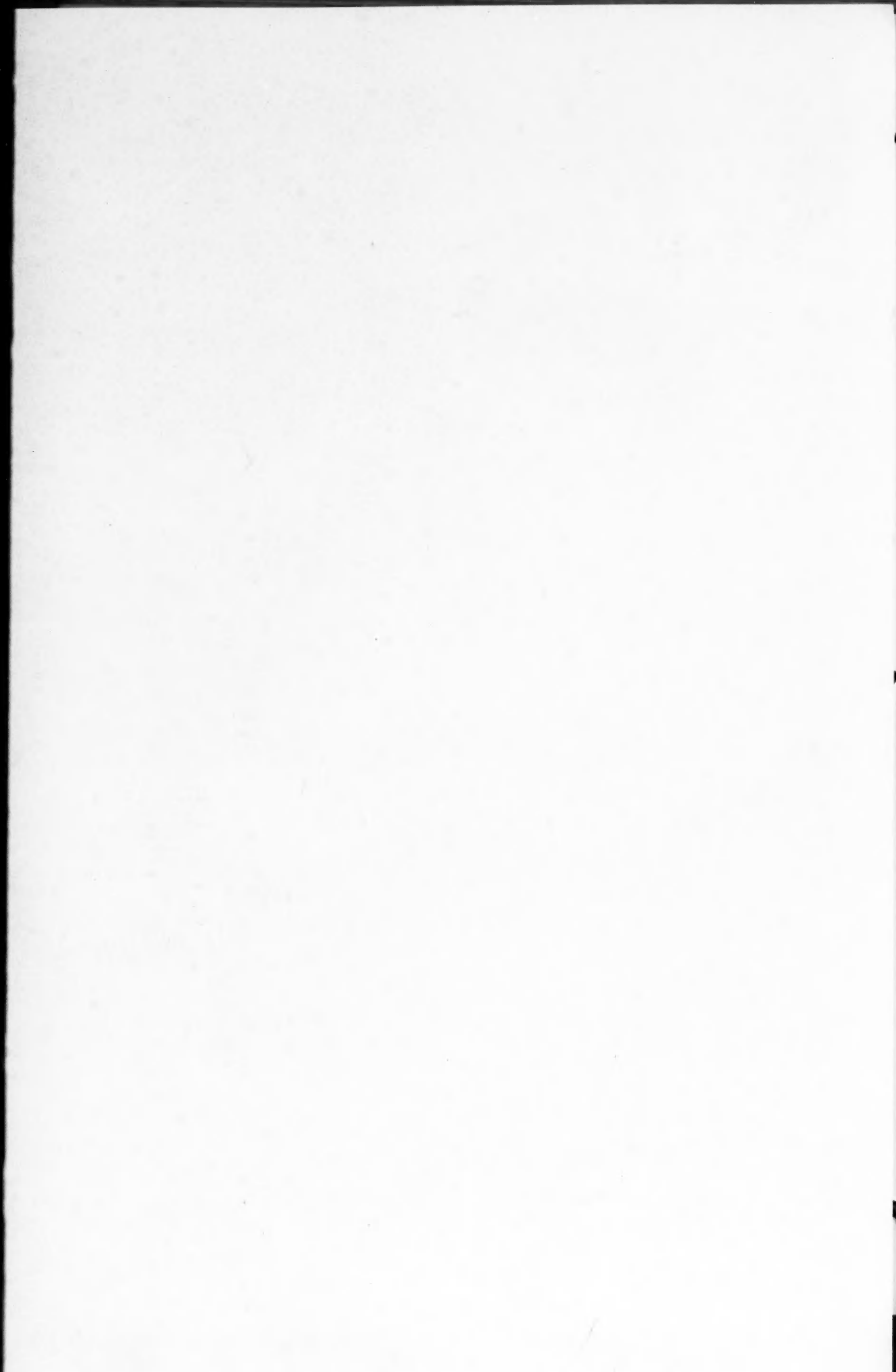
In evaluating the potential usefulness of paper chromatography, there are certain characteristics which must be kept in mind. The procedure is simple. Satisfactory resolutions can be obtained with a minimum of equipment and in a short time. Under the proper conditions, resolution of mixtures can be effected which cannot be readily separated by other methods. Only a few micrograms of material are employed. This is a definite disadvantage if a sufficient amount of a component is desired for further characterization. A large series of identical chromatograms can be run and the resulting components pooled, but this is tedious at best. Since the R_f values are, in effect, constants under a given set of conditions, it is an elegant procedure for qualitative analysis. However, because of the small amount of material, quantitative analyses with a precision of $\pm 5\%$ can only be accomplished under the most exacting conditions. By suitable modification of the solvent system and other factors, almost any mixture can be successfully resolved.

In presenting this brief summary, no attempt has been made to present details of specific procedures. Such information has been summarized in several excellent monographs and books. Block, Durrum, and Zweig (2), Brimley and Barrett (4), and Lederer and Lederer (5) are especially recommended.

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METHODS FOR THE SEPARATION OF ORGANIC COMPOUNDS BY PAPER CHROMATOGRAPHY

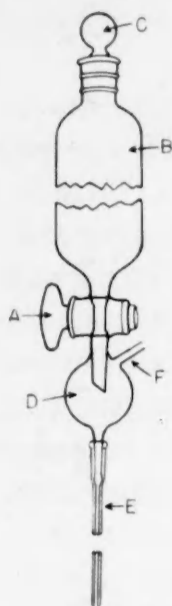
(Summary of a few selected procedures. For details, see Refs. 2, 4, and 5)

Type of compound	Derivative	Common solvent system	Color indicator
Alcohols.....	Xanthogenates	$n\text{-C}_4\text{H}_9\text{OH} : 2\% \text{ aq. KOH}$	Nanitropusside + hydroxylamine HCl
	3,5-Dinitrobenzoates	$\text{C}_7\text{H}_{16} : \text{CH}_3\text{OH}$	1-Naphthylamine
	3,6-Dinitrophthalates	$\text{iso-C}_6\text{H}_{11}\text{OH} : \text{NH}_4\text{OH} : \text{H}_2\text{O}$	NaOH + ethyl acetoacetate
Phenols.....	Methylolphenols	$n\text{-C}_4\text{H}_9\text{OH} : \text{NH}_4\text{OH}$	p -Nitrobenzenediazonium fluoborate
	Phenylazobenzenesulfonates	$\text{sec.-C}_4\text{H}_9\text{OH} : \text{aq. Na}_2\text{CO}_3$	(Derivative is colored)
Aliphatic acids			
Volatile ($\text{C}_1\text{-C}_9$)	Hydroxamates	$n\text{-C}_6\text{H}_{11}\text{OH} : \text{HCOOH} : \text{H}_2\text{O}$	FeCl_3
H_2O -sol., non-vol.	_____	$\text{C}_2\text{H}_5\text{OH} : \text{NH}_4\text{OH} : \text{H}_2\text{O}$	Thymol blue
α -Keto acids	2,4-Dinitrophenyl hydrazones	$\text{tert.-C}_5\text{H}_{11}\text{OH} : \text{C}_2\text{H}_5\text{OH} : \text{H}_2\text{O}$	NaOH
Higher fatty acids	Hydroxamates	$\text{*CH}_3\text{COOC}_2\text{H}_5 : \text{tetrahydrofuran} : \text{H}_2\text{O}$	FeCl_3
Aromatic acids.....	_____	$n\text{-C}_4\text{H}_9\text{OH} : (\text{NH}_4)_2\text{CO}_3 : \text{H}_2\text{O}$	Methyl red
	2,4-Dinitrophenyl hydrazones	$\text{*}(\text{C}_2\text{H}_5)_2\text{O} : \text{pet. ether}$	NaOH
	_____	$\text{C}_2\text{H}_5\text{OH} : \text{pet. ether}$	
Carbohydrates.....	_____	$\text{C}_6\text{H}_5\text{OH} : \text{H}_2\text{O}$	{ 3,4-Dinitrobenzoic acid
	_____	$n\text{-C}_4\text{H}_9\text{OH} : \text{CH}_3\text{COOH} : \text{H}_2\text{O}$	{ Aniline + phthalic acid
Amines.....	_____	$n\text{-C}_4\text{H}_9\text{OH} : \text{CH}_3\text{COOH} : \text{H}_2\text{O}$	{ 1-Naphthol
	_____	$\text{C}_6\text{H}_5\text{OH} : \text{H}_2\text{O}$	Triketohydrindene (ninhydrin)
Amino acids.....	_____	2,6-lutidine:2,4,6-collidine: H_2O	Triketohydrindene (ninhydrin)
	_____	$n\text{-C}_4\text{H}_9\text{OH} : \text{CH}_3\text{COOH} : \text{H}_2\text{O}$	
	Dinitrophenyl amino acids	$n\text{-C}_4\text{H}_9\text{OH} : \text{H}_2\text{O}$	(Derivative is colored)

*Reversed-phase chromatography.

AN AUTOMATIC CONSTANT-RATE ADDITION FUNNEL

By WILBERT J. HUMPHLETT*



An addition funnel has been devised for delivery of liquids at a constant rate *without requiring the attention of the operator*. The manipulation necessary to regulate the rate of flow of liquids from the usual dropping funnel is familiar to the chemist. This flow varies with the head of the liquid in the funnel and with the constraint developed by the stopcock as the stopcock grease dissolves or melts. Dropping funnels which employ greaseless Teflon stopcocks or which operate without a stopcock, such as the device of Hershberg (1), nevertheless require adjustment as the liquid head changes.

The constant-rate addition funnel described here operates from a constant head of liquid, simply provided by employment of the principle of the office-type, inverted "water cooler." The rate of flow is controlled by the flow from the constant head through a capillary of sufficient diameter of bore. A set of capillaries to choose from accomplishes this purpose rapidly. The usual dropping funnel (with a standard taper No. 8, or larger, stopcock) can be converted cheaply to this device by a glass blower.

To operate, the stopcock, *A* (see the figure), is closed and the funnel reservoir, *B*, is filled with liquid. A stopper, *C*, is inserted, and the stopcock is opened wide. Liquid floods the constant-head chamber, *D*, up to the tip of the funnel. It maintains this level and flows no higher. (The office "water-cooler" does not run over.) Loss of liquid from *D* through the capillary, *E*, is replenished by flow from the funnel reservoir, *B*. Atmospheric pressure is maintained in *D* by an air-inlet tube, *F*; a partial vacuum forces air bubbles into the funnel reservoir, *B*, with each small addition of liquid to *D*.

For dimensions, a typical laboratory funnel has a standard taper 29/42 stopper and a 1-liter, cylindrical reservoir (a cylindrical shape allows use of heating tape for maintaining preheated temperatures or melting a solid). A No. 8 stopcock is required, since smaller bores do not allow free flow of bubbles into the funnel reservoir. The constant-head chamber is of 100-ml. capacity, with a standard taper 10/30 joint for connection to the capillary. The tip of the funnel extends into the constant-head chamber a distance equal to two thirds of the chamber diameter; the funnel tip has a beveled edge. Employment of capillaries of 15-cm. length and $\frac{1}{4}$ - to $1\frac{1}{4}$ -mm. bore allows addition of 1 liter of many liquids of varying viscosities during $\frac{1}{2}$ to 3 hours.

Reference

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